Biochem. J. (1986) 240, 691–698 (Printed in Great Britain)

Carbohydrate structures of the third component of rat complement

Presence of both high-mannose and complex type oligosaccharide chains

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We investigated the carbohydrate structure of the third component of complement (C3) newly synthesized by cultured rat hepatocytes. When the cells were incubated with [^{3}H]mannose, [^{3}H]galactose or [^{3}H]glucosamine, these radioactive precursors were incorporated only into the α subunit of C3, demonstrating that only the α subunit contains oligosaccharide chains. [^{3}H]Mannose-labelled C3 was purified from the culture medium by immunoaffinity chromatography. Oligosaccharides prepared by Pronase digestion and strong alkaline hydrolysis were separated into two fractions by Bio-Gel P-2 chromatography (Fractions I and II). The two fractions were analysed by concanavalin A–Sepharose chromatography, ion-exchange high-performance liquid chromatography, and Bio-Gel P-4 gel filtration before and after sequential exoglycosidase digestions. It was found that Fraction I contained two complex type oligosaccharide chains, $(NeuAc)_2(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2$ and $(NeuAc)_3(Gal)_3(GlcNAc)_3(Man)_3(GlcNAc)_2$, and Fraction II contained the high-mannose type, consisting mainly of $(Man)_8(GlcNAc)_2$. Taken together with the carbohydrate composition of rat serum C3, the results suggest that rat C3 has one high-mannose type oligosaccharide chain and two complex type chains in the α subunit, which is different from the proposal for human C3.

INTRODUCTION

The third component of complement (C3) plays a principal role in the complement system by participating in both the classical and alternative pathways of complement activation (Müller-Eberhard, 1975; Reid & Porter, 1981). C3 is the most abundant of all the complement proteins, and is synthesized mainly by hepatocytes (Alper *et al.*, 1969; Brade *et al.*, 1977), although it is also produced by cells of the monocyte/macrophage series (Stecher & Thorbecke, 1967; Bentley *et al.*, 1976). The protein is a glycoprotein of M_r 187000, composed of two disulphide-linked subunits, α and β with M_r 115000 and 75000, respectively (Bokisch *et al.*, 1975). It has been established that C3 is initially synthesized as a single polypeptide chain precursor (pro-C3), which is subsequently converted to the mature form by selective proteolysis (Brade *et al.*, 1977; Patel & Minta, 1979). This conversion takes place intracellularly just before secretion (Misumi *et al.*, 1984).

Previous evidence indicates that human C3 contains a carbohydrate moiety in both subunits (Taylor *et al.*, 1977; Tack *et al.*, 1979). The carbohydrate composition analysis showed that *N*-acetylglucosamine and mannose, amounting to 1.7% by weight, were the only monosaccharides detected in human C3 (Tomana *et al.*, 1985). Structural analysis of the oligosaccharide chains of human C3 has most recently demonstrated that both the subunits contain only the high-mannose type, $(Man)_{5-9}(GlcNAc)_2$ (Hase *et al.*, 1985; Hirani *et al.*, 1986). In the present paper, we describe the structural analysis of the *N*-linked oligosaccharide chains of rat C3, demonstrating that only α subunit contains oligosaccharide chains, which are composed of both the high-mannose and complex types, in contrast with those in human C3.

MATERIALS AND METHODS

Materials

[2-^{3}H]Mannose (27.2 Ci/mmol), [6-^{3}H]galactose (29.2 Ci/mmol), [1,6-^{3}H]glucosamine (39.3 Ci/mmol) and [^{35}S]methionine (1100 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.). Eagle's minimum essential medium (MEM) was obtained from Nissui Seiyaku (Tokyo, Japan); goat anti-(rat C3) serum from Cappel Laboratories (West Chester, PA, U.S.A.); leupeptin, pepstatin, antipain, chymostatin, elastinal and phosphamidon from Protein Research Foundation (Osaka, Japan); concanavalin A (Con A)–Sepharose (8 mg/ml of gel) from Pharmacia Fine Chemicals (Uppsala, Sweden); Bio-Gel P-2 (200–400 mesh), P-4 (under 400 mesh) and P-6 (200–400 mesh) from Japan Bio-Rad Laboratories (Tokyo, Japan). The sources of enzymes were as follows: collagenase (*Clostridium histolyticum*) from Wako Junyaku (Osaka, Japan); Pronase (*Streptomyces griceus*) from Calbiochem–Behring (La Jolla, CA, U.S.A.); neuraminidase (*Arthrobacter ureafaciens*) from Nakarai Chemicals (Kyoto, Japan); β-galactosidase (*Charonia lampas*), β-*N*-acetylhexosaminidase (jack bean) and α-mannosidase (jack bean) from Seikagaku Kogyo (Tokyo, Japan).

Abbreviations used: C3, the third component of complement; Con A, concanavalin A; MEM, minimum essential medium; P_i/NaCl, phosphate-buffered saline (0.01 M-phosphate/0.15 M-NaCl, pH 7.5).

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Hepatocyte culture and metabolic labelling

Hepatocytes were isolated from adult male Wistar rats, weighing 200–250 g, by the collagenase perfusion method (Seglen, 1976). Isolated hepatocytes were cultured in Falcon dishes as described previously (Oda & Ikehara, 1982). For labelling carbohydrate moieties, cells were incubated with [^{3}H]mannose, [^{3}H]glucosamine, or [^{3}H]galactose (200 μCi/dish) in low-glucose MEM (0.56 mM-glucose and 10 mM-pyruvate) for 8–24 h (for mannose) or 6 h (for galactose and glucosamine). For labelling polypeptide chains, cells were incubated with [^{35}S]methionine (100 μCi/dish) in MEM lacking unlabelled methionine for 6 h. After incubation, medium was separated and centrifuged at 10000 *g* for 10 min, followed by immunoprecipitation or immunoaffinity chromatography to obtain the labelled C3.

Immunoprecipitation and SDS/polyacrylamide gel electrophoresis

To the culture medium obtained as above, the following proteinase inhibitors were added: phenylmethanesulphonyl fluoride (final concentration 2 mM) and a mixture of leupeptin, pepstatin, antipain, chymostatin, elastinal and phosphamidon (final concentration 10 μg of each/ml). Immunoprecipitation of C3 was performed with carrier C3 as described previously (Oda *et al.*, 1983). The immunoprecipitates were analysed by electrophoresis on SDS/polyacrylamide gels (7.5%, w/v) according to Laemmli (1970). Gels were fixed and subjected to fluorography as described previously (Oda *et al.*, 1983).

Immunoaffinity purification of [^{3}H]mannose-labelled C3

Medium obtained after incubating cells with [^{3}H]mannose was mixed with proteinase inhibitors as above and applied to an anti-(rat C3) IgG–Sepharose column (1 cm × 4 cm) prepared as described previously (Ikehara *et al.*, 1981). The column was washed with 50 ml of P_i/NaCl and subsequently with 300 ml of 0.01 M-phosphate buffer containing 0.5 M-NaCl. ^{3}H-labelled C3 was eluted with 3 M-NaSCN, pooled and dialysed at 4 °C against P_i/NaCl and then against distilled water. The sample was concentrated to an appropriate volume by a Speed-Vac Concentrator (Sarvant Instruments, Hicksville, NY, U.S.A.), and finally lyophilized.

Preparation of glycopeptides and oligosaccharides

Lyophilized [^{3}H]mannose-labelled C3 (1×10^6 d.p.m.) was dissolved in 0.5 ml of 0.1 M-Tris/HCl (pH 8.0)/2 mM-$CaCl_2$ containing 5 mg of Pronase, and incubated at 60 °C under a toluene atmosphere (Carlsson & Stigbrand, 1984). Fresh Pronase (5 mg) was added after 24 h of incubation. After 48 h of total incubation the sample was heated at 100 °C for 3 min and applied to a Bio-Gel P-6 column (1.6 cm × 94 cm) equilibrated with 0.1 M-NH_4HCO_3. After elution with the same solution, radioactive fractions were pooled and concentrated. After desalting by passing through a Bio-Gel P-2 column (1.6 cm × 90 cm) radioactive glycopeptides thus obtained were lyophilized.

The lyophilized glycopeptides were dissolved in 0.2 ml of 1 M-NaOH/4 M-$NaBH_4$, and heated at 80 °C for 24 h (Zinn *et al.*, 1978). After cooling, the sample was neutralized with a few drops of acetic acid and desalted through the Bio-Gel P-2 as above. Oligosaccharides thus obtained were lyophilized and *N*-acetylated with acetic anhydride in 0.4 ml of saturated $NaHCO_3$ (Takasaki & Kobata, 1978), followed by desalting as above.

Exoglycosidase digestions

Oligosaccharides thus prepared were sequentially digested with the following exoglycosidases under the indicated conditions at 37 °C. Neuraminidase, 0.1 unit in 100 μl of 0.1 M-sodium acetate (pH 5.0) for 24 h; β-galactosidase, 0.2 unit in 150 μl of 0.1 M-citrate/phosphate (pH 3.6) for 48 h; β-*N*-acetylhexosaminidase, 1.5 units in 200 μl of 0.1 M-sodium citrate (pH 5.0) for 48 h; and α-mannosidase, 2.5 units in 300 μl of 0.1 M-sodium citrate (pH 4.5) for 48 h. Digestions were terminated by heating the samples at 100 °C for 3 min, and each sample was desalted as above.

Con A–Sepharose affinity chromatography

A Con A–Sepharose column (0.6 cm × 8 cm) was equilibrated with 10 mM-Tris/HCl (pH 8.0) containing 150 mM-NaCl, 1 mM-$CaCl_2$ and 1 mM-$MgCl_2$ at room temperature. A sample dissolved in 0.2 ml of the above buffer was applied to the column, which was washed with 12 ml of the same buffer. Elution was performed with 15 ml of 10 mM-α-methyl D-glucoside in the buffer at room temperature, followed by 15 ml of 100 mM-α-methyl D-mannoside at 60 °C (Cummings & Kornfeld, 1982). Fractions (0.5 ml) were collected and assayed for radioactivity. Radioactive fractions were pooled, desalted and subjected to gel filtration through a Bio-Gel P-4 column.

Bio-Gel P-4 chromatography

Sizes of oligosaccharides were determined by Bio-Gel P-4 chromatography (Yamashita *et al.*, 1982). A lyophilized sample was dissolved in 0.4 ml of a mixture of glucose oligomers prepared by partial hydrolysis of dextran, and applied to a Bio-Gel P-4 column (2 cm × 100 cm) maintained at 55 °C. Fractions (1.0 ml) were collected. Radioactive peaks obtained were compared with elution positions of glucose oligomers, which were detected by a refractometer. Sizes of oligosaccharides were expressed as glucose units.

Ion-exchange h.p.l.c.

H.p.l.c. was carried out by essentially the same method as that of Baenziger & Natowicz (1981). Lyophilized oligosaccharides were dissolved in 25 mM-KH_2PO_4 (pH 4.0) and injected into a MicroPak AX-10 column (Varian Associates; 0.4 cm × 30 cm) equilibrated with the same buffer. The column was eluted with the same buffer for 15 min followed by an increasing linear gradient of 25–500 mM-KH_2PO_4 (pH 4.0) for 30 min at a flow rate of 1.0 ml/min. Fractions (0.3 ml) were collected and assayed for radioactivity.

Carbohydrate composition analysis of rat serum C3

Rat serum C3 was purified from freshly prepared serum by using the immunoaffinity column as described previously (Ikehara *et al.*, 1981). Sialic acid was

determined according to Warren (1959). Determination of neutral and amino sugars was performed by g.l.c. as described previously (Ikehara *et al.*, 1981).

Autolytic fragmentation of C3

C3 which had been labelled with either [^{35}S]methionine, [^{3}H]mannose, [^{3}H]galactose or [^{3}H]glucosamine was isolated from the culture medium by immunoprecipitation. The immunoprecipitates were immediately dissolved in 50 μl of 10 mM-Tris/HCl (pH 7.5) containing 1% (w/v) SDS and heated at 100 °C for 10 min for autolytic fragmentation of C3 (Sim & Sim, 1981). The samples were analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions as described above.

RESULTS

Carbohydrate composition of rat serum C3

Rat serum C3 purified by immunoaffinity chromatography, when analysed by SDS/polyacrylamide-gel electrophoresis, showed a typical pattern of the α and β subunits, corresponding to M_r 115000 and 65000, respectively. Thus, the molecular mass of rat C3 was estimated to be 180000. The purified serum C3 was analysed for carbohydrate composition. As shown in Table 1, C3 was found to contain mannose, galactose, glucosamine, sialic acid and a trace amount of fucose, amounting to about 4% by weight. The presence of galactose and sialic acid residues suggest that rat C3 contains the complex type oligosaccharide chains, in contrast with those in human C3 (Tomana *et al.*, 1985). In addition, a relatively high content of mannose residues suggests the presence of high-mannose or hybrid type oligosaccharides.

SDS/polyacrylamide-gel electrophoresis of the newly synthesized C3

To investigate the carbohydrate structure in the following experiments, we took advantage of a culture system of rat hepatocytes for preparation of the labelled C3. Metabolically labelled C3 was purified from the culture medium and analysed by SDS/polyacrylamide-gel electrophoresis under reducing conditions (Fig. 1). [^{35}S]Methionine-labelled C3 showed the subunit structure of α and β (lane 1). However, when the cells were labelled with [^{3}H]mannose, [^{3}H]glucosamine, or [^{3}H]galactose, only the α subunit, but never the β subunit, was labelled with these precursors (lanes 2–4). These results indicate that rat C3 has oligosaccharide chains only in the α subunit, in contrast with the evidence that human C3 has the carbohydrate moieties in both the subunits (Taylor *et al.*, 1977; Tack *et al.*, 1979).

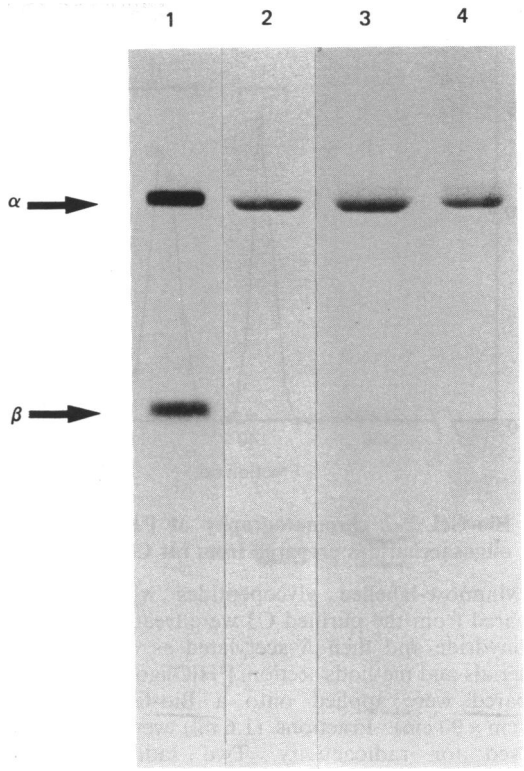

Fig. 1. SDS/polyacrylamide-gel electrophoresis of the metabolically labelled C3

Rat hepatocytes were cultured in the presence of either [^{35}S]methionine, [^{3}H]mannose, [^{3}H]galactose, or [^{3}H]glucosamine as described in the Materials and methods section. The newly synthesized and secreted C3 was isolated from the culture medium by immunoprecipitation, and analysed by electrophoresis on SDS/polyacrylamide gels (7.5%, w/v) followed by fluorography. Lane 1, [^{35}S]methionine-labelled C3; lane 2, [^{3}H]mannose-labelled C3; lane 3, [^{3}H]galactose-labelled C3; lane 4, [^{3}H]glucosamine-labelled C3. Symbols α and β indicate the α and β subunits, respectively, of C3.

Table 1. Carbohydrate composition of rat serum C3

The purified rat serum C3 was analysed for carbohydrate composition as described in the Materials and methods section. An M_r of 180000 was used for calculation of residues/molecule.

Carbohydrate	Content (g/100 g of protein)	Content (residues/molecule)
Fucose	0.06	0.6
Mannose	1.16	11.6
Galactose	0.59	5.9
Glucosamine	1.08	11.0
Sialic acid	1.08	6.2
Total weight (%)	3.97	

Structural analysis of oligosaccharide chains

We used [^{3}H]mannose-labelled C3 for preparation of oligosaccharide chains, since the mannose residue is a component commonly found in both the high-mannose and complex type oligosaccharide chains. [^{3}H]Mannose-labelled C3 was digested with Pronase and the resultant glycopeptides were subjected to alkaline borohydride treatment. When the oligosaccharides thus prepared were applied to a Bio-Gel P-2 column, two peaks of radioactivity were obtained (Fig. 2). These peaks, designated as Fractions I and II, were separately pooled, and subjected to the following analyses.

Analysis of Fraction I. When a portion of Fraction I was subjected to h.p.l.c. through a MicroPak AX-10 column with an ion-exchange system, several peaks were

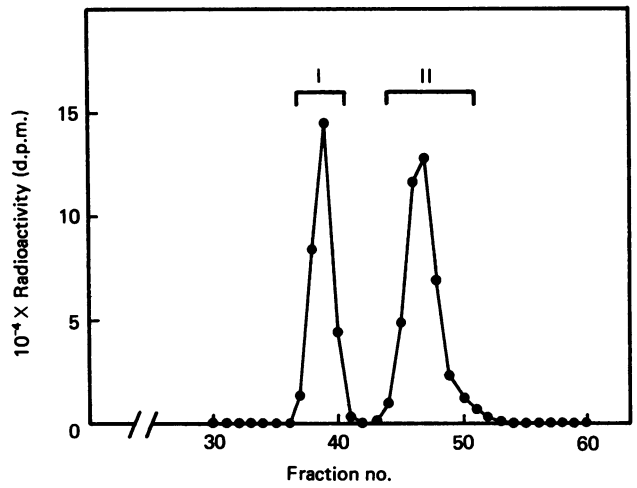

Fig. 2. Bio-Gel P-2 chromatography of [^{3}H]mannose-labelled oligosaccharides prepared from rat C3

[^{3}H]Mannose-labelled glycopeptides which had been prepared from the purified C3 were treated with alkaline borohydride and then *N*-acetylated as described in the Materials and methods section. [^{3}H]Oligosaccharides thus prepared were applied onto a Bio-Gel P-2 column (1.6 cm × 90 cm). Fractions (1.6 ml) were collected and assayed for radioactivity. Two radioactivity peaks were separately pooled and designated as Fractions I and II.

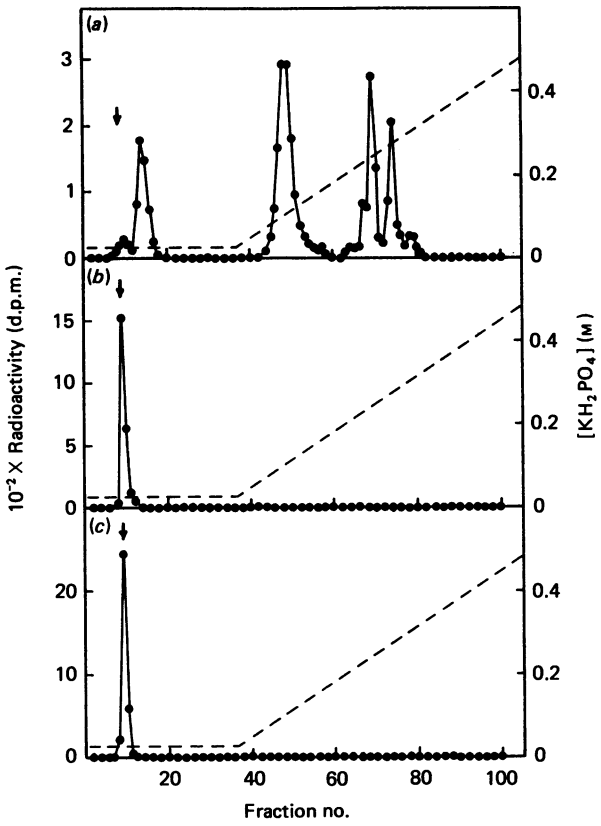

Fig. 3. Separation of [^{3}H]mannose-labelled oligosaccharides of C3 by h.p.l.c. on an anion-exchange column

Portions of Fractions I and II obtained in Fig. 2 were injected onto a MicroPak AX-10 column (0.4 cm × 30 cm). The column was eluted at a flow rate of 1.0 ml/min with 25 mM-KH_2PO_4 (pH 4.0) for 15 min followed by an increasing linear gradient of 25–500 mM-KH_2PO_4 (pH 4.0) for 30 min (broken line). Fractions (0.3 ml) were collected and assayed for radioactivity (solid line). (*a*) Fraction I; (*b*) the sample after treatment of Fraction I with neuraminidase; (*c*) Fraction II. An arrow indicates the elution position of neutral oligosaccharides.

obtained, as shown in Fig. 3(*a*). Under the conditions used, it is known that neutral oligosaccharides are eluted first, followed by sequential elution of anionic oligosaccharides according to their negative charges, which are mainly due to the number of sialic acid residues (Baenziger & Natowicz, 1981). When the same sample was digested with neuraminidase and subjected to h.p.l.c., all the major peaks found in Fig. 3(*a*) disappeared and shifted to the neutral position (Fig. 3*b*), indicating that Fraction I contains sialylated oligosaccharides.

Since Fraction I was suggested to contain the complex type oligosaccharides, we subjected this fraction to Con A–Sepharose chromatography after neuraminidase treatment. As shown in Fig. 4, we obtained two major peaks, the flow-through fraction (Fraction I-A) and a fraction eluted with 10 mM-α-methyl D-glucoside (Fraction I-B), and a minor peak eluted with 100 mM-α-methyl D-mannoside (Fraction I-C). The two major fractions separated by Con A–Sepharose chromatography were further analysed by gel permeation chromatography through a Bio-Gel P-4 column before and after sequential treatments with exoglycosidases. Fig. 5 shows the results obtained with Fraction I-A. The sample before exoglycosidase treatment was eluted at the position corresponding to 16 glucose units (Fig. 5*a*). After sequential treatments with exoglycosidases, oligosaccharides with the following sizes were obtained: 13 glucose units with β-galactosidase; 7 units with β-*N*-acetylhexosaminidase; and finally two components with 5 and 1 unit(s) after α-mannosidase treatment (Figs. 5*b*–5*d*). A possible structure of the oligosaccharide in Fraction I-A deduced from these results is a tri-antennary complex type with $(Gal)_3(GlcNAc)_3(Man)_3(GlcNAc)_2$.

Fraction I-B was also analysed by chromatography on the same column (Fig. 6). The original sample was eluted at the position corresponding to 13 glucose units (Fig. 6*a*). Sequential digestions with exoglycosidases finally yielded two components with 5 and 1 glucose unit(s) (Figs. 6*b*–6*d*). The results suggest that Fraction I-B contains a bi-antennary complex type oligosaccharide chain consisting of $(Gal)_2(GlcNAc)_2(Man)_3(GlcNAc)_2$. Possible structures proposed for these oligosaccharides in Fraction I-A and I-B are in good agreement with their chromatographic behaviours through Con A–Sepharose (Fig. 4); the tri-antennary type flows through the column, while the bi-antennary type is eluted from the column with 10 mM-α-methyl D-glucoside (Cummings & Kornfeld, 1982).

Analysis of Fraction II. When Fraction II was analysed by h.p.l.c. on a MicroPak AX-10 column, the radioactivity peak was obtained only at the elution position corresponding to that of neutral oligosaccharides (Fig. 3*c*). In addition, when subjected to Con

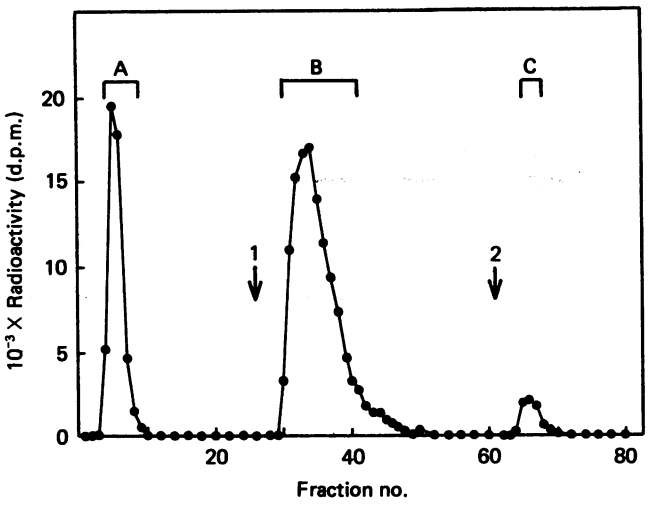

Fig. 4. Con A–Sepharose chromatography of Fraction I after neuraminidase treatment

Fraction I was treated with neuraminidase and fractionated by affinity chromatography on a Con A–Sepharose column (0.6 cm × 8 cm) as described in the Materials and methods section. Arrows indicate the starting positions of stepwise elution: 1, with 10 mM-α-methyl D-glucoside; 2, with 100 mM-α-methyl D-mannoside. Fractions (0.5 ml) were collected and assayed for radioactivity. Radioactivity peaks A, B and C were separately pooled.

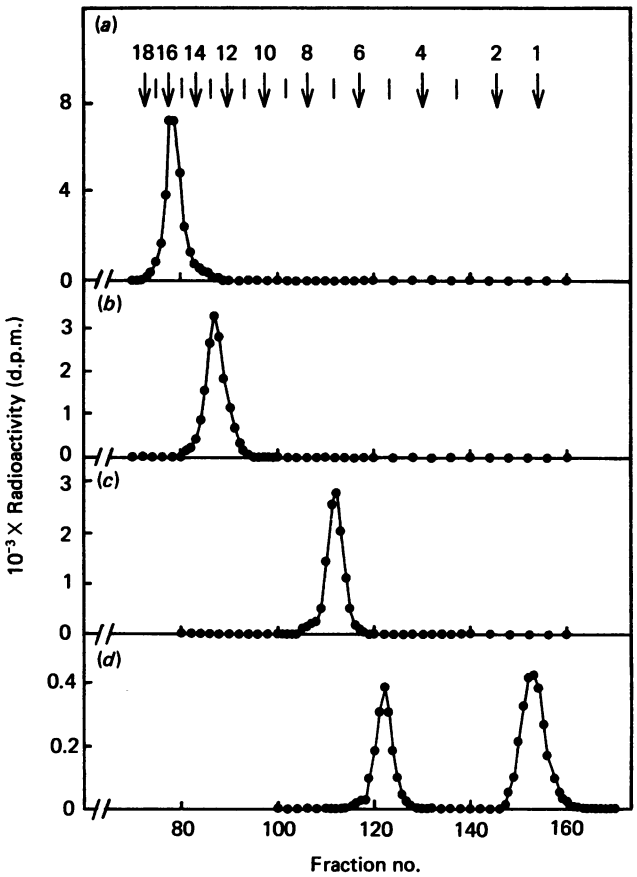

Fig. 5. Bio-Gel P-4 chromatography of Fraction I-A before and after sequential exoglycosidase digestions

Fraction I-A obtained in Fig. 4 before and after exoglycosidase digestions was mixed with glucose oligomers and fractionated by chromatography on a Bio-Gel P-4 column (2 cm × 100 cm). Fractions (1.0 ml) were collected and assayed for radioactivity. Arrows with numbers indicate elution positions of the respective glucose oligomers (glucose units) used as internal standards. (*a*) Fraction I-A before treatment; (*b*) the sample after digestion of Fraction I-A with β-galactosidase; (*c*) the sample after digestion of (*b*) with β-*N*-acetylhexosaminidase; (*d*) the sample after digestion of (*c*) with α-mannosidase.

A–Sepharose chromatography, all the radioactivity in Fraction II was eluted from the column with 100 mM-α-methyl D-mannoside (results not shown). These results suggest that Fraction II contains oligosaccharides with the high-mannose type or hybrid type lacking sialic acid residues. To clarify this point, we analysed this fraction by chromatography on the Bio-Gel P-4 column before and after treatment with α-mannosidase (Fig. 7). The sample before treatment was eluted at the position with 12 glucose units (Fig. 7*a*), while the sample after α-mannosidase digestion was eluted as two peaks, a minor peak at 5 glucose units and a major peak at 1 glucose unit (Fig. 7*b*). The results indicate that Fraction II contains the high-mannose type oligosaccharide chains, consisting mainly of 8 mannose residues. This is based on the finding that the original sample with 12 glucose units was converted to one component with 5 glucose units (possibly Man-GlcNAc-GlcNAc) and the other as free mannose after α-mannosidase treatment. However, oligosaccharides with 7 or 9 mannose residues may be present because the elution profile obtained by Bio-Gel P-4 chromatography was rather broad (Fig. 7*a*).

Taken together with the carbohydrate composition of serum C3 (Table 1), these results suggest that rat C3 has one high-mannose type oligosaccharide chain and two complex types with different side chains in the α subunit.

Carbohydrate attachment sites in the α subunit

Possible carbohydrate attachment sites have recently been predicted in human and mouse C3, based on their amino acid sequences deduced from the nucleotide sequences of cDNAs (DeBruijn & Fey, 1985; Lundwall *et al.*, 1984; Wetsel *et al.*, 1984). However, no such data concerning rat C3 are available at present. We attempted to determine the approximate attachment sites of three oligosaccharide chains in the α subunit, for which we took advantage of autolytic fragmentation of C3 (Sim & Sim, 1981).

C3 which had undergone autolysis was reduced and analysed by SDS/polyacrylamide-gel electrophoresis (Fig. 8). [^{35}S]Methionine-labelled C3 underwent autolysis under denaturing conditions, resulting in two additional fragments with M_r 73000 and 42000 (lane 2). The available evidence indicates that the former is the *C*-terminal fragment (αC) of the α subunit and the latter is its *N*-terminal portion (αN), because autolysis occurs at the thiol ester site of the α subunit (Sim & Sim, 1981). When C3 labelled with [^{3}H]mannose, [^{3}H]galactose or [^{3}H]glucosamine was autolysed under the same conditions as above, all the ^{3}H-labelled precursors were detected in the αN fragment as well as in the uncleaved α subunit (Fig. 8, lanes 3–5). The results clearly indicate that

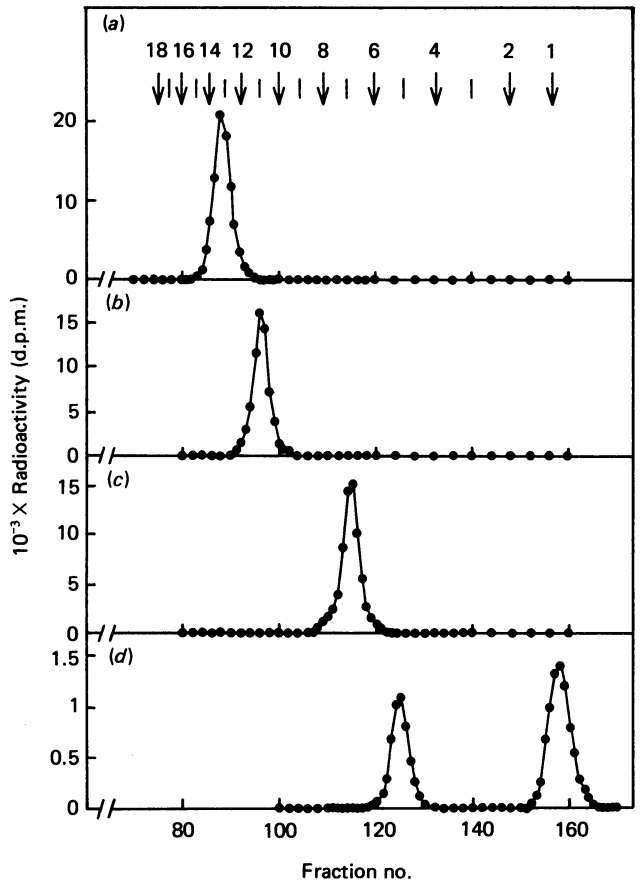

Fig. 6. Bio-Gel P-4 chromatography of Fraction I-B before and after sequential exoglycosidase digestions

Fraction I-B obtained in Fig. 4 was sequentially digested with exoglycosidases as in Fig. 5. Samples before and after treatments (*a*)–(*d*) were analysed by chromatography on the Bio-Gel P-4 column under the same conditions as in Fig. 5.

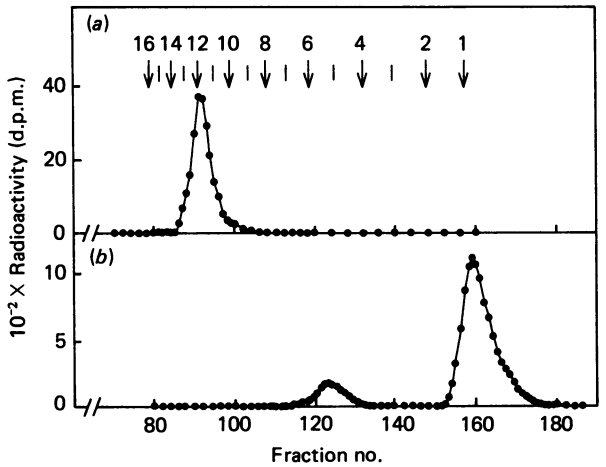

Fig. 7. Bio-Gel P-4 chromatography of Fraction II before and after α-mannosidase digestion

Fraction II obtained in Fig. 2 was analysed by Bio-Gel P-4 chromatography before (*a*) and after (*b*) digestion with α-mannosidase. All the conditions used were the same as those described in Fig. 5.

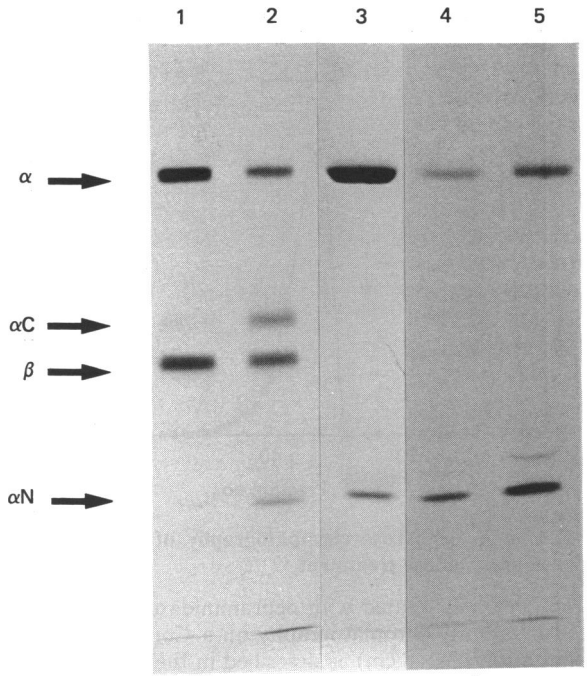

Fig. 8. SDS/polyacrylamide-gel electrophoresis of C3 before and after autolytic fragmentation

Metabolically labelled C3 was immunoprecipitated from freshly prepared culture medium. The immunoprecipitates were dissolved in 10 mM-Tris/HCl (pH 7.5) containing 1% (w/v) SDS and 1% (v/v) 2-mercaptoethanol and heated at 100 °C for 1 min (lane 1), or dissolved in the same buffer containing 1% SDS and heated at 100 °C for 10 min (lanes 2–5). Samples were analysed by SDS/polyacrylamide gel electrophoresis under reducing conditions followed by fluorography. Lanes 1 and 2, [^{35}S]methionine-labelled C3; lanes 3–5, [^{3}H]mannose-, [^{3}H]galactose- and [^{3}H]glucosamine-labelled C3, respectively. α and β denote the α and β subunits, respectively, of C3; αN and αC represent the *N*- and *C*-terminal fragments, respectively, of the α subunit obtained after autolytic fragmentation of C3.

all the three oligosaccharide chains are located at the *N*-terminal portion of the α subunit. Since rat C3a has no Asn-Xaa-Ser/Thr sequence (Jacobs *et al.*, 1978), carbohydrate attachment sites must be in the portion between the *N*-terminus of C3α′ (i.e. the α subunit of C3b) and the thiol ester site.

DISCUSSION

In the present study rat C3 was metabolically labelled with [^{35}S]methionine or [^{3}H]sugars in the hepatocyte culture system, and isolated by one step of immunoprecipitation or immunoaffinity chromatography. The simple and rapid purification avoided a possible degradation of C3, which is quite susceptible to various proteinases (Müller-Eberhard, 1975).

Labelling of C3 with [^{3}H]mannose, [^{3}H]galactose or [^{3}H]glucosamine demonstrated that rat C3 has the carbohydrate moieties only in the α subunit but not in the β subunit, which is in contrast with the evidence that human C3 has oligosaccharide chains in both the subunits (Taylor *et al.*, 1977; Tack *et al.*, 1979). [^{3}H]Mannose-labelled oligosaccharides prepared from rat C3 were

separated into two fractions by Bio-Gel P-2 chromatography. The two fractions (Fractions I and II) were characterized by anion-exchange chromatography, Con A–Sepharose chromatography and finally gel permeation chromatography before and after sequential exoglycosidase digestions. The results presented demonstrated that Fraction I contains two complex type oligosaccharides with the tri- and bi-antennary side chains, while Fraction II contains only the high-mannose type. Most of the complex type oligosaccharides are suggested to be sialylated (Table 1, Figs. 3*a* and 3*b*). On the basis of these results we propose that rat C3 has three oligosaccharide chains with different structures consisting of $(NeuAc)_3$-$(Gal)_3(GlcNAc)_3(Man)_3(GlcNAc)_2$, $(NeuAc)_2(Gal)_2$-$(GlcNAc)_2(Man)_3(GlcNAc)_2$ and $(Man)_8(GlcNAc)_2$. These structures of rat C3 are in contrast to those proposed for human C3, all of which are the high-mannose type oligosaccharides; the α subunit contains mainly $(Man)_{8-9}(GlcNAc)_2$ and the β subunit has $(Man)_{5-6}(GlcNAc)_2$ (Hase *et al.*, 1985; Hirani *et al.*, 1986).

Possible carbohydrate attachment sites have been predicted in human and mouse C3 based on the Asn-Xaa-Ser/Thr triplets in the primary structures deduced from the respective cDNAs. Three potential binding sites in human C3 are located at amino acid residues 228 and 946 in the α subunit and at amino acid 63 in the β subunit (DeBruijn & Fey, 1985). Mouse C3 has two possible attachment sites in the α subunit at amino acid residues 169 and 947 but no sites in the β subunit (Lundwall *et al.*, 1984; Wetsel *et al.*, 1984). However, no data are available for the primary structure of rat C3 from which we can predict possible attachment sites of three oligosaccharide chains. To know approximate attachment sites of the carbohydrate moieties in rat C3, we utilized a characteristic of C3, autolytic fragmentation (Sim & Sim, 1981), and examined the distribution of radioactivity of ^3H-labelled sugars in its autolytic fragments. Autolytic fragmentation under the conditions used resulted in production of two additional fragments (αN and αC) derived from the α subunit. All the radioactive sugars were detected only in the αN fragment with M_r 42000. The results indicate that all the three oligosaccharide chains are located in the *N*-terminal portion of the α subunit. These locations are in contrast with those sites found in mouse and human C3, in which one binding site is located near the *C*-terminus of the α subunit (Wetsel *et al.*, 1984; DeBruijn & Fey, 1985). Our attempt to demonstrate where individual oligosaccharide chains are localized within the αN fragment was not successful, because we could not succeed in further fragmentation of the αN.

Asparagine-linked oligosaccharides are synthesized and processed during intracellular transport of glycoproteins by a well-defined pathway (Kornfeld & Kornfeld, 1985). Most glycoproteins found in serum contain only complex type oligosaccharides, although membrane proteins usually contain both high-mannose and complex types (Pollack & Atkinson, 1983). The presence of both types, however, has been reported as rare cases in secreted glycoproteins such as IgM (Cahour *et al.*, 1984), IgE (Baenziger & Kornfeld, 1974*a*,*b*), and apolipoprotein B (Vauhkonen *et al.*, 1985). Rat C3 as presented here is another case of this class. The available information on oligosaccharide type, i.e. complex or high-mannose, with regard to its position in the polypeptide chain indicates that, in general, complex type oligosaccharides are located towards the *N*-terminus of the polypeptide and that high-mannose types are located farther towards the *C*-terminus (Pollack & Atkinson, 1983). It seems likely that the final oligosaccharide form depends on steric hindrance, since at least some glycosyltransferases as well as glycosidases act after folding of the protein has taken place (Kornfeld & Kornfeld, 1985). Such evidence indicates that the protein primary structure can determine whether complex or high-mannose oligosaccharides will occur and that protein conformation affects processing of high-mannose oligosaccharides.

We thank Drs. K. Yamashita (Kobe University School of Medicine) and K. Oda (our Department) for useful suggestions and discussions. This work was supported in part by grants from the Ministry of Education, Science and Culture of Japan.

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Received 11 June 1986; accepted 18 August 1986